Proc. Nat. Acad. Sci. USA
Vol. 71, No. 11, pp. 4429–4434, November 1974

On the Chemical Nature of Transfer Factor

(immune response/leukocyte extract/transfer of immunological information/double-stranded RNA/guinea pig)

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Communicated by J. D. Watson, April 3, 1974

ABSTRACT Two transfer factors prepared in an experimental animal model, the guinea pig, have been tested for their susceptibility to various enzymes of known specificity. The biological activity of these immune response mediators can be destroyed by RNase III, an enzyme that degrades duplex RNA. It, therefore, appears that these transfer factors consist entirely or partly of double-stranded RNA.

Transfer factor is a puzzling immunological phenomenon. It appears, to those who have observed the phenomenon, to be a transfer of immunological information from a population of "educated" leukocytes to a population of "naive" leukocytes. A subcellular, leukocyte component is involved, and it is generally believed that information for specific immune responses is transferred.

The transfer factor phenomenon can perhaps be most easily grasped by considering a specific example, such as the cell-mediated immune response mounted against the small chemical hapten, dinitrochlorobenzene (DNCB). DNCB is a reactive chemical that permeates into the tissues and conjugates to the lysine and cysteine residues of various proteins, causing them to appear foreign. A naive individual who is exposed to DNCB will destroy the foreign material in a slow and moderate reaction. During this *primary response* another important event occurs: there is a mobilization of a specific part of the immune system. Thus, if the individual ever encounters the same antigen again, he will mount a *secondary response* that is both more rapid and more forceful than the primary response.

The cornerstone of the transfer factor phenomenon, as contained in the work of Lawrence (1) and of Jeter, Tremaine, and Seebohm (2), is the statement that the leukocytes of the immunologically experienced individual can be made to yield a subcellular component (the transfer factor) that can transmit to a naive individual the immunological information necessary for the mobilization of a specific part of the immune system. Thus, the naive individual, upon receipt of the leukocyte extract, becomes immediately able to mount a strong *secondary* response upon his *first* exposure to the antigen.

Since the discovery of transfer factor was an unexpected addition to the field of immunology, its very existence has posed several interesting questions. What are these subcellular leukocyte components that can apparently substitute for antigen in the mobilization of the immune system? Where do the transfer factors function in the development of the full immune response, and why have these intermediate information carriers been designed into the system? Are they representative of a more general mechanism of information transfer between cells?

The chemical nature of transfer factor has been a problem of long-standing interest. Our knowledge in this area is just developing and includes four facts, secured by Lawrence and his colleagues. The biologically active material is small enough to pass through a dialysis membrane (3); thus, transfer factor is too small to be or to code for any of the proteins involved in a specific immune response. Furthermore, transfer factor can withstand treatment with DNase, pancreatic RNase, and trypsin (4).

In this paper we present the results of an enzymological analysis of two transfer factors prepared in the guinea pig experimental system. The data indicate that the biological activity of these transfer factors resides entirely or partly in species of low molecular weight, double-stranded RNA. The implications of this result in terms of the possible mode of action of transfer factor will be discussed.

Preparation of transfer factors

The experimental system we use is the guinea pig, and the immune response we have studied is the delayed hypersensitivity reaction. In the companion to this paper (5) we described procedures that allowed us to make 20 preparations of biologically active transfer factor, amidst 65 failures. These transfer factors were of two types: one primes an animal to give a secondary response to dinitrochloro-benzene (DNCB) and the other primes the recipient to respond to *ortho*-chlorobenzoylchloride (OCBC). In brief, the preparation of these transfer factors involved (*a*) sensitizing the donor animals with antigen on the ear, thus provoking a primary response, (*b*) waiting 7 days and then challenging the animals with antigen on the flank, eliciting the delayed hypersensitivity response (Fig. 1), (*c*) sacrificing the donor animals on day 11 so that lymphoid cells from the spleen, lungs, and peritoneal exudate could be obtained, and (*d*) recovering a dialyzable component from these cells which, when injected into a naive animal would allow him to immediately mount a delayed hypersensitivity response upon his first exposure to the antigen (Fig. 2).

Experimental design

The general design of the experiments reported here involves the treatment of transfer factor with enzymes of known specificity before the material is injected into a naive recipient. The subsequent response of the recipient upon challenge with antigen will show whether or not the ability of the material

Abbreviation: DNCB, dinitrochlorobenzene.

to transmit the delayed hypersensitivity capacity has been destroyed by the enzyme.

At the outset it may be well to consider the possible pitfalls of the enzymatic approach. First, when exposing a transfer factor preparation to an enzyme one must explicitly determine that the enzyme is working; the current methods for preparing transfer factor lead to the accumulation of considerable amounts of salt capable of inhibiting most enzymes. Second, even if the transfer factor preparation were to survive a given enzyme such as DNase, this does not necessarily mean that transfer factor is devoid of DNA. For instance, bacteriophage lambda has an essential DNA component, but it would survive DNase digestion because of the protection provided by a protein coat.

Similarly, resistance to pancreatic RNase does not guarantee that single-stranded RNA is absent from transfer factor. The RNA might be protected—or it could be exposed but lacking in cytidylic acid and uridylic acid residues.

Resistance to protease digestion must also be considered as a yardstick of uncertain length: some proteins are known to be resistant to protease digestion (6).

Suppose a transfer factor preparation were to be destroyed by a highly purified enzyme: does this mean that transfer factor has been chemically identified? Not necessarily. One must consider the possibility that there are impurities in the enzyme preparation. Furthermore, the transfer factor, although inactivated by one enzyme, might still contain additional components that are integral parts of the biologically active compound.

All this notwithstanding, the enzymatic approach is invaluable for three reasons. First, if successful, it allows one to conclude that the activity of transfer factor resides entirely or partly in a definable chemical species. Second, one can hope to reach this conclusion using amounts of material that are well below the levels required for direct chemical analysis. Third, one is able to study preparations of material that are not highly purified.

Test substrates

To monitor the effectiveness of the enzyme digestions, radioactive test substrates were prepared. These included tritium-labeled bacteriophage T7 DNA, phosphorus-labeled single-stranded and double-stranded RNA, and sulfur-labeled *Escherichia coli* proteins, as described below.

Preparation of Radioactive DNA. A thymine-requiring strain of *E. coli* was grown in medium containing tritiated thymidine and infected with bacteriophage T7 until lysis. The phage were precipitated with polyethylene glycol (7), resuspended, and centrifuged to equilibrium in CsCl. The phage peak was identified by plaque formation. The viral DNA was then extracted by adding sodium lauroyl-sarcosinate to 1% and heating the solution to 50° for 5 min. CsCl centrifugation was used to purify the radioactive viral DNA.

Preparation of Single-Stranded RNA. Purified *E. coli* RNA polymerase containing the sigma subunit was used to transcribe T7 DNA *in vitro.* [^{32}P]ATP was used to label the product RNA. The reaction mixture is described in ref. 8.

Preparation of Double-Stranded RNA. An artificial RNA molecule, available from Miles Laboratories, was used as template; it consisted of a random sequence of adenylic, cytidylic, and uridylic acid residues. The complementary strand was synthesized by the QB replicase (9–11), which is able to initiate synthesis on stretches of poly(C) in the template. [^{32}P]UTP was used to label the product. After 45 minutes of reaction, about 20% of the single-stranded template had been converted to a duplex state; all of the incorporated radioactivity was located in these duplex portions, as shown by the resistance of this radioactive material to pancreatic RNase, unless the material was first heated to 100° and then rapidly cooled.

Preparation of Radioactive Proteins. E. coli was grown in the presence of [^{35}S]sulfate. The crude cell lysate was fractionated by ammonium sulfate precipitation, chromatography on DEAE–cellulose, and sedimentation through glycerol (12). The ^{35}S-labeled proteins were a gift of Robert Horvitz.

FIGS. 1–7. Transfer factors are subcellular leukocyte components that appear to be able to transmit information for specific immune responses from *experienced* leukocytes to *naive* leukocytes. This paper describes the results of an enzymological analysis of two transfer factors prepared in the guinea pig experimental system. These transfer factors carry information for cell-mediated responses against the antigens dinitrochlorobenzene (DNCB) and *ortho*-chlorobenzoylchloride (OCBC).

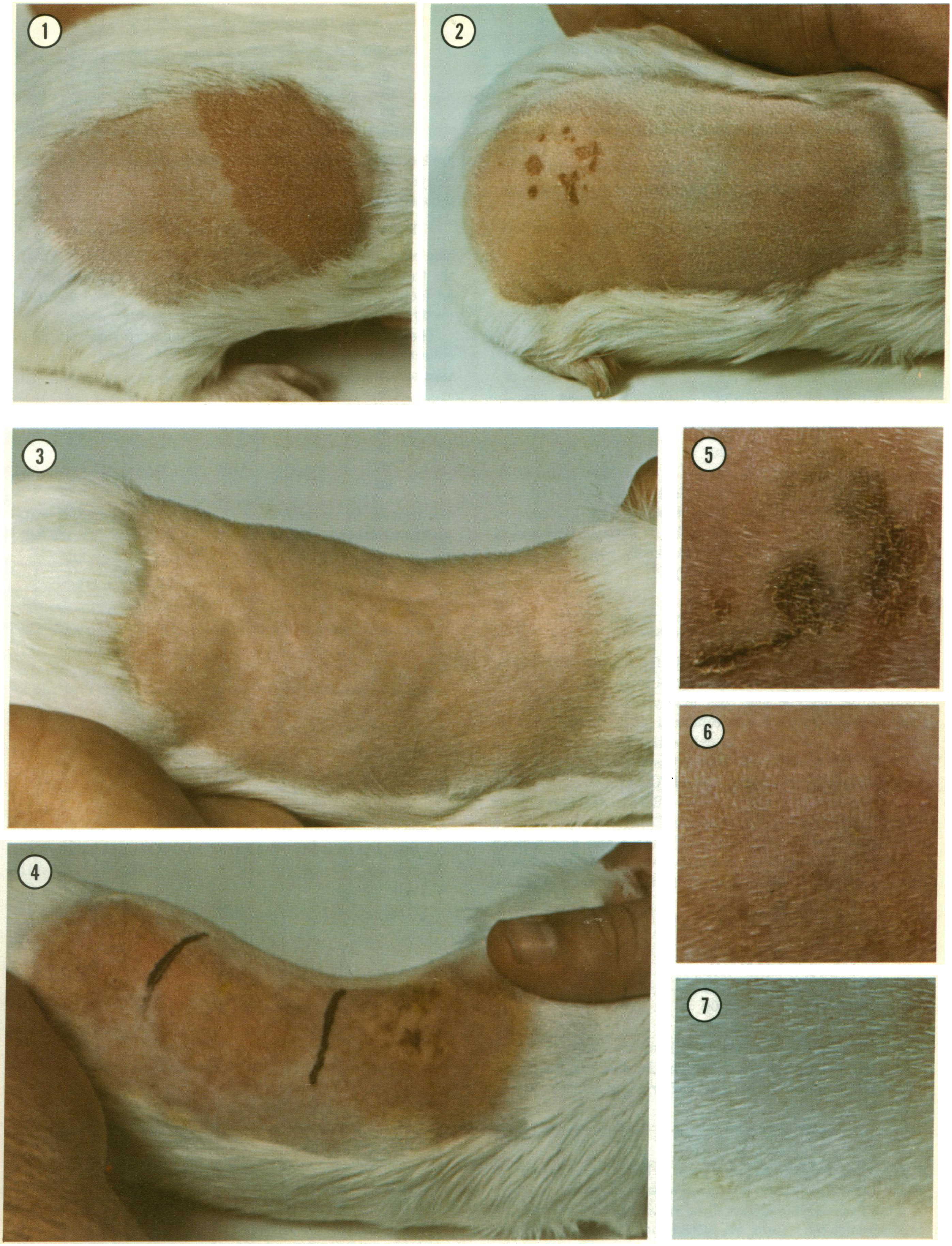
FIG. 1. Immune capacity developed in an animal that has been directly exposed to antigen. Six days after DNCB or OCBC was painted on the ear, the back of the animal was shaved and the antigen was applied again. This provoked the secondary response (a delayed hypersensitivity reaction) shown. The reaction is designated +2 in severity; it is characterized by a homogeneous erythema (redness), which represents an increased blood supply in the area where responding leukocytes are eliminating the antigen. The lymphoid tissue of animals such as that shown in Fig. 1 serves as the source of transfer factor (5).

FIG. 2. An example of the immunity acquired by a naive animal that received an injection of transfer factor (about 50% of the material from a single donor). The transfer factor was injected into the peritoneal cavity, and 48 hr later the animal was shaved and challenged (5) with two concentrations of antigen: 35 μl of 50 mM DNCB (on the lower flank) and 35 μl of 20 mM DNCB (on the upper flank). Beginning about 15 hr later, the animal showed a strong delayed hypersensitivity response. The reaction at the site that received the lower concentration of antigen was a mild erythema. At the site that received the higher concentration of antigen, the reaction was more severe (+4) and was characterized by a patchy necrosis in addition to erythema and induration (swelling). Necrosis represents a generalized tissue destruction in the area where the antigen is being eliminated.

FIG. 3. An example of an animal unable to respond to challenge with antigen because the transfer factor he received had been treated with RNase III, an enzyme that specifically degrades double-stranded RNA.

FIG. 4. Treatment of transfer factor with DNase, RNase, or protease prior to injection has little effect on the biological activity of the material. Here, 48 hr after injection of the enzymatically treated transfer factor, the test animal was shaved and challenged with three concentrations of antigen: 35 μl of 50 mM DNCB (upper flank), 25 mM DNCB (middle flank), and 5 mM DNCB (lower flank). The response to the highest concentration of antigen is +4 in severity, as in Fig. 2. The two vertical black lines were made by a magic marker to separate the challenge sites.

FIGS. 5–7. Transfer factor is heat-sensitive. A solution of DNCB transfer factor heated to 80° retained full biological activity (Fig. 5); a solution heated to 85° retained partial biological activity (Fig. 6); a solution heated to 90° was inactivated (Fig. 7).



FIGS. 1–7. *(Legend appears at bottom of the previous page.)*

Transfer factor is resistant to deoxyribonuclease

Transfer factor was first tested for its susceptibility to inactivation by DNase. The following experiment was done. An amount of transfer factor capable of transmitting a +4 delayed hypersensitivity capacity to a naive animal (generally the material from 1/3 of a donor) was diluted 20-fold, so as to reduce the concentration of salts that might interfere with the enzyme digestion. The dilution buffer was 10 mM Tris, pH 8–5 mM $MgSO_4$, and the final NaCl concentration was about 0.15 M. Pancreatic DNase (Worthington, Code DPFF) was then added to 20 μg/ml, and the reaction mixture (10 ml) was incubated for 1 hr at 25°.

To make sure that the DNase was working, we added as a test substrate tritium-labeled bacteriophage T7 DNA (final concentration: 0.5 μg/ml of DNA; 63,000 cpm/ml). Virtually all of the test DNA was degraded within 60 min but the

Minutes	cpm/0.1 ml‡	Activity
0	6290*	+4
10	1340	
30	540	
60	20	+4

biological activity of the transfer factor was unaffected and gave responses identical to those shown in Fig. 4. Digestion of the biologically active material by as little as a factor of two would be expected to give a markedly weaker response, as shown by dilution experiments (5).

Transfer factor is resistant to pancreatic RNase and T1 RNase

The transfer factor was next tested for its susceptibility to pancreatic RNase, which hydrolyzes single-stranded RNA chains after uridylic acid and cytidylic acid residues. The transfer factor solution was diluted 1:10 with 10 mM Tris, pH 8, and 1 mM EDTA to reduce the NaCl concentration to 0.3 M. The solution was then exposed to 20 μg/ml of pancreatic RNase (Worthington, Code RASE) for 60 min at 37°.

To make sure that the pancreatic RNase was working, we added 26,000 cpm/ml of radioactive T7 mRNA that had been synthesized *in vitro* with purified T7 DNA and *E. coli* RNA polymerase. Almost all of the test RNA was degraded in 60 min but the biological activity of the transfer factor remained

Minutes	cpm/0.1 ml‡	Activity
0	2630*	+4
10	110	
30	110	
60	100	+4

intact and gave responses like those shown in Fig. 4.

In a parallel experiment, T1 ribonuclease, which specifically cuts single-stranded RNA chains after guanylic acid residues, was used. Again the test RNA was degraded in 60 min but

Minutes	cpm/0.1 ml‡	Activity
0	1200*	+4
10	190	
30	140	
60	150	+4

the biological activity of the transfer factor survived, and the delayed hypersensitivity responses were the same as those shown in Fig. 4.

Transfer factor is resistant to protease digestion

The enzymatic approach was next extended to ask whether the transfer factor could be destroyed by Pronase. The transfer factor solution was diluted 20-fold as before, and digested for 60 min with 300 μg/ml of self-digested (13) Pronase.

The test substrate in the reaction mixture consisted of a collection of radioactive *E. coli* proteins, at a final concentration of 0.4 μg/ml; they were totally degraded in 60 min but

Minutes	cpm/0.1 ml‡	Activity
0	16,760*	+4
10	6,360	
30	410	
60	40	+3 to +4

the transfer factor solution was virtually undiminished in its ability to transfer delayed hypersensitivity capacity, mediating reactions identical to those shown in Fig 4.

Transfer factor is destroyed by RNase III

In contrast to its resistance to DNase, pancreatic and T1 RNase, and Pronase, transfer factor activity is destroyed by RNase III. This enzyme specifically degrades double-stranded RNA (14, 15). The experiment is as follows. The transfer factor solution was diluted 20-fold with 50 mM Tris, pH 8, and the following reagents were added: $MgCl_2$ to 10 mM, 2-mercaptoethanol to 1 mM, and glycerol to 5% (v/v).

As a test substrate, radioactive double-stranded RNA was prepared; this was done by incubating a random ACU polymer with the Qβ replicase and radioactive RNA precursors. The double-stranded product RNA was added to the transfer factor solution (0.2 μg/ml), followed by RNase III. The enzyme was a highly purified preparation (15) generously given to us by Dr. Robert Crouch of the NIH. The RNase III not only degraded the test substrate but also destroyed the

Minutes	cpm/0.1 ml‡	Activity
0	35,000*	+4
10	9,750	
30	480	
60	60	0

transfer factor activity, giving the response shown in Fig. 3. This result has been obtained with two independent preparations of both DNCB and OCBC transfer factor.

Can we be certain that the activity that destroyed the transfer factor was RNase III, and not some impurity in the enzyme preparation—for instance, an unseen enzyme directed against glycoproteins or phospholipids? If RNase III is indeed the enzyme that degrades the transfer factor, then it should be possible to protect the transfer factor from digestion by adding an excess of nonradioactive, double-stranded RNA to the reaction mixture. We have done this experiment, using nonradioactive poly(rA·rU) and poly(rI·rC) in a 100-

* There was virtually no loss of cpm or biological activity in the parallel reaction mixture incubated without enzyme.

‡ For each enzyme digestion the amount of test substrate still in macromolecular form was determined by precipitating 1% of the sample in trichloroacetic acid.

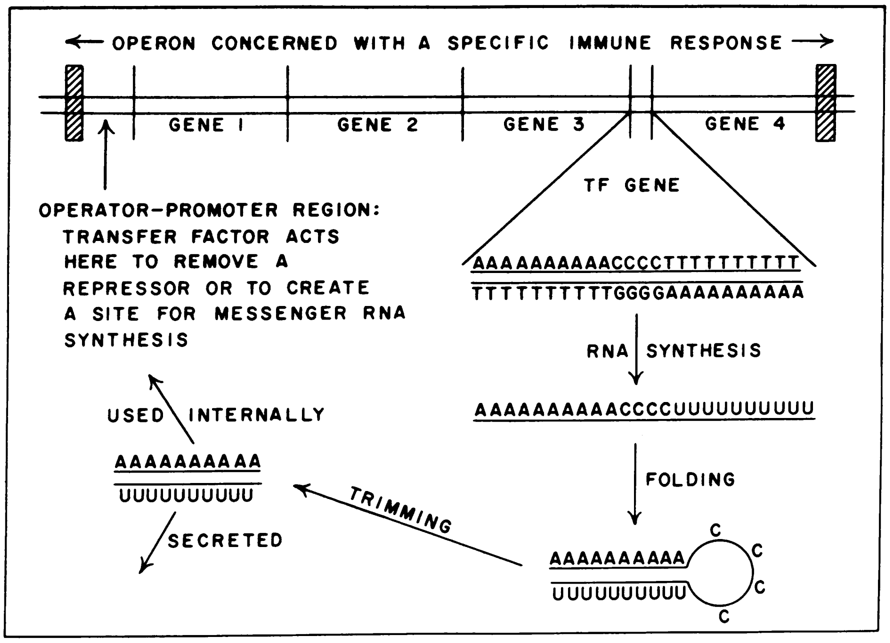


FIG. 8. A diagram showing how transfer factor might work. The data of the paper support the structure in the *lower left*, where transfer factor is depicted as a small, double-stranded RNA molecule.

fold excess over the radioactive test substrate. As expected, digestion of the radioactive test substrate did not occur

Minutes	cpm/0.1 ml‡	Activity
0	20,020	+4
10	19,800	
30	20,600	
60	20,100	+4

and the transfer factor activity was protected, giving rise to the responses shown in Fig. 4.†

This result argues strongly that the RNase III is the enzyme that is responsible for the destruction of the transfer factor, and that, therefore, transfer factor consists partly or entirely of double-stranded RNA.

Heat lability of the transfer factor

To test for the heat sensitivity of the transfer factor solution, aliquots were diluted 20-fold with 50 mM Tris, pH 8, and then incubated for 10 min at various temperatures, ranging from 50° to 100°C. The solutions were then injected into test animals which, after 48 hr, were challenged with DNCB.

The solutions incubated at 80° or less retained full transfer factor activity (Fig. 5). Temperatures above 90° destroyed the activity (Fig. 7). The solutions treated at 85° had an intermediate level of transfer factor activity, as judged by the strength of the delayed hypersensitivity reactions they mediated (Fig. 6). This heat sensitivity is consistent with the melting of a double-stranded nucleic acid.

Discussion

The simplest interpretation of the data is that transfer factor is a small, double-stranded RNA molecule. This conclusion is based on the relative resistance of transfer factor to several enzymes: DNase, pancreatic and T1 RNase, and Pronase, and its sensitivity to RNase III. Whether the nucleic acid component of transfer factor functions alone, or in conjunction with an additional unidentified component, remains unknown.

One possibility for such an additional component would be the presence of antigen itself coupled to the transfer factor. This has long been considered as an explanation for the transfer factor phenomenon (16, 17). The hypothesis proposes that antigen coupled with transfer factor is more highly immunogenic than antigen alone. Thus, the transfer factor phenomenon would always appear to be present in a preparation in which the level of antigen itself was too low to be detected. The "superantigen hypothesis" remains viable, not only because it is reasonable, but also because it is extremely difficult to prove that antigen is *not* present in the transfer factor preparation. For instance, in one experiment Lawrence, Rappaport, Converse, and Tillett demonstrated transfer factor activity in a preparation directed against a histocompatibility antigen that would be too large to pass through a dialysis membrane (16). But the devil's advocate position here would be that a small antigenic determinant derived from the original antigen was the moiety associated with the transfer factor. In another experiment Burger, Vetto, and Malley demonstrated that transfer factor was not retained on a column that contained antibodies known to be directed against the antigen (17). But the objection could still be made that the antigen moiety is protected within the complex and, thus, was unable to bind to the column. If antigen is responsible for the specificity of transfer factor, one might picture a mechanism in which the nucleic acid component functions as carrier to introduce the antigen to the responding leukocytes. These cells, using a special cell surface receptor that recognizes the nucleic acid component, might then be able to more rapidly and efficiently make contact with the antigen. Alter-

† Could the +4 reaction here be due to the added poly (rA·rU) and poly (rI·rC)? No, because unlabeled double-stranded RNA was also added to the previous RNase III digestion mixture, but after the reaction was over. It produced no response.

natively, the transfer factor might stimulate leukocytes that were independently interacting with antigen.

If the "transfer factor + antigen = superantigen" formulation is not correct, then one must explain how a double-stranded RNA molecule of low molecular weight could by itself enable a population of leukocytes to develop an immune response against a specific antigen. The transfer factor is too small (it can pass through a dialysis membrane) to code for the specific proteins involved in an immune response. It could, however, stimulate a leukocyte by functioning as a *negative* control element or depressor, a general idea that draws upon the studies of gene control in *E. coli* (23, see also ref 18). One might picture the double-stranded RNA molecule as competing with a DNA sequence in the leukocyte for the attention of a specific repressor. If the repressor has a stronger affinity for the entering transfer factor than for its natural operator, the operon would be derepressed and transcribed. This would lead to the production of the components involved in the immune response.

Alternatively, the transfer factor could be viewed as functioning as a *positive* control element. In this situation, which could be more economical, the transfer factor would act directly and positively by matching its nucleic acid sequence with a specific immune DNA sequence so as to activate the region for transcription.

A characteristic of both of these models for transfer factor function is that the immune system is essentially preprogrammed and specific genes need only to be turned on (Fig. 8).

Considering the present data it is also possible to consider the way in which the transfer factor itself might be produced. Rather than exist as a self-replicating molecule, the transfer factor could be the product of transcription from one element of the operon (see Fig. 8). We would picture a region in the operon that contains an inverted and symmetric base sequence so that its transcript could self-anneal to form a hairpin structure. Removal of the single-stranded region(s) would yield active transfer factor which could then exert its influence internally or upon other leukocytes.

The involvement of RNA in the transmission of immunological information is a problem of general current interest. Fishman and Adler (19) have demonstrated that peritoneal exudate cells from rats, incubated *in vitro* with bacteriophage T2, can yield an RNA extract that elicits antibodies against T2 from a culture of normal lymphoid cells. A similar phenomenon has been described by Askonas and Rhodes (20), using hemocyanin in a mouse system *in vitro*. They interpreted their results as indicating that the RNA functions as a carrier for residual antigen, greatly enhancing its immunogenicity. However, Dray, Bell, Fishman, and Adler (21, 22) believe that this cannot be the full explanation. In extending the phenomenon to rabbits, they found that the allotype of the antibodies elicited by the RNA extract was that of the donor, not the recipient. From this result, they concluded that the RNA might include intact messenger RNA molecules which can be taken up and used by recipient cells. The large size of the RNA involved in these experiments, as well as its sensitivity to pancreatic RNase, distinguish this material from transfer factor.

Although the transfer factor phenomenon is described here in terms of one experimental system, the differentiating leukocyte, it might have further implications in developmental biology. Perhaps other types of cell–cell interactions leading to differentiation also involve the transmission of information by a small molecule such as transfer factor.

Drs. Albert Coons and James Watson provided thoughtful readings of the manuscript. We are also indebted to Dr. Charles Dressler who reminded us at a key moment that guinea pigs need vitamin C. This work was supported by the National Institutes of Health (GM 17088) and the American Cancer Society (NP-57C). D.D. is a recipient of a Public Health Service Research Career Development Award (GM 70440). S.R. is an undergraduate in Harvard College and has received support from the Camille and Henry Dreyfus Fund.

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